TITLE OF THE INVENTION
HCV RNA-DEPENDENT RNA POLYMERASE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Application No. 60/535,708, filed January 9, 2004, which is hereby incorporated by reference herein.

BACKGROUND OF THE INVENTION

The references cited in the present application are not admitted to be prior art to the claimed invention.

It is estimated that about 3% of the world's population are infected with the Hepatitis C virus (HCV). (Wasley *et al.*, 2000. *Semin. Liver Dis. 20*, 1-16.) Exposure to HCV results in an overt acute disease in a small percentage of cases, while in most instances the virus establishes a chronic infection causing liver inflammation and slowly progresses into liver failure and cirrhosis. (Iwarson, 1994. *FEMS Microbiol. Rev. 14*, 201-204.) Epidemiological surveys indicate HCV plays an important role in hepatocellular carcinoma pathogenesis. (Kew, 1994. *FEMS Microbiol. Rev. 14*, 211-220, Alter, 1995. *Blood 85*, 1681-1695.)

The HCV genome consists of a single strand RNA about 9.5 kb in length, encoding a precursor polyprotein about 3000 amino acids. (Choo *et al.*, 1989. *Science 244*, 362-364, Choo *et al.*, 1989. *Science 244*, 359-362, Takamizawa *et al.*, 1991. *J. Virol. 65*, 1105-1113.) The HCV polyprotein contains the viral proteins in the order: C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B.

Individual viral proteins are produced by proteolysis of the HCV polyprotein. Host cell proteases release the putative structural proteins C, E1, E2, and p7, and create the N-terminus of NS2 at amino acid 810. (Mizushima *et al.*, 1994. *J. Virol. 68*, 2731-2734, Hijikata *et al.*, 1993. *Proc. Natl. Acad. Sci. USA 90*, 10773-10777.)

The non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B presumably form the virus replication machinery and are released from the polyprotein. A zinc-dependent protease associated with NS2 and the N-terminus of NS3 is responsible for cleavage between NS2 and NS3. (Grakoui *et al.*, 1993. *J. Virol. 67*, 1385-1395, Hijikata *et al.*, 1993. *Proc. Natl. Acad. Sci. USA 90*, 10773-10777.)

A distinct serine protease located in the N-terminal domain of NS3 is responsible for proteolytic cleavages at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions. (Barthenschlager *et al.*, 1993. *J. Virol. 67*, 3835-3844, Grakoui *et al.*, 1993. *Proc.*

Natl. Acad. Sci. USA 90, 10583-10587, Tomei *et al.*, 1993. *J. Virol. 67*, 4017-4026.) RNA stimulated NTPase and helicase activities are located in the C-terminal domain of NS3.

NS4A provides a cofactor for NS3 protease activity. (Failla *et al., J. Virol.* 1994. *68*, 3753-3760, De Francesco *et al.,* U.S. Patent No. 5,739,002.)

NS5A is a highly phosphorylated protein conferring interferon resistance. (Pawlotsky 1999. *J. Viral Hepat. Suppl. 1*, 47-48.)

NS5B provides an RNA-dependent RNA polymerase. (De Francesco *et al.,* International Publication Number WO 96/37619, published November 28, 1996, Behrens *et al.,* 1996. *EMBO 15*, 12-22, Lohmann *et al.,* 1998. *Virology 249*, 108-118.) Soluble RNA-dependent RNA polymerase can be produced by a 21 amino acid truncation at the C terminus. (Yamashita *et al., The Journal of Biological Chemistry 273*:15479-15486, 1998, Ferrari *et al., Journal of Virology 73*:1649-1654, 1999.)

Different genotypes and quasispecies of HCV have been identified. (Farci *et al., Seminars in Liver Disease 20*:103-126, 2000, Okamoto *et al., Virology 188*:331-341, 1992.)

SUMMARY OF THE INVENTION

The present invention features NS5B polypeptides from different clinically important HCV genotypes. The polypeptides can be used individually, or as part of a panel of RNA-dependent RNA polymerases, to evaluate the effectiveness of a compound to inhibit NS5B activity.

Thus, a first aspect of the present invention describes a purified polypeptide comprising an amino acid sequence selected from the group consisting of: SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5. A "purified polypeptide" is present in an environment lacking one or more other polypeptides with which it is naturally associated and/or is represented by at least about 10% of the total protein present.

In different embodiments, the purified polypeptide represents at least about 50%, at least about 75%, or at least about 95% of the total protein in a sample or preparation. Reference to "purified polypeptide" does not require that the polypeptide has undergone any purification and may include, for example, chemically synthesized polypeptide that has not been purified.

Another aspect of the present invention describes a recombinant nucleic acid comprising a nucleotide sequence encoding an amino acid sequence selected from the group consisting of: SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5. A recombinant nucleic acid is nucleic acid that by virtue of its sequence and/or form does not occur in nature. The form of the nucleic acid is provided by its association with other nucleic

acids found in nature, such the absence of one or more other nucleic acid regions naturally associated with a particular nucleic acid (*e.g.*, upstream or downstream regions) and/or purified nucleic acid.

Another aspect of the present invention describes a method of evaluating the ability of a compound to inhibit HCV RNA-dependent RNA polymerase. The method involves measuring the ability of the compound to inhibit the activity of one or more HCV RNA-dependent RNA polymerases having an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5.

Unless particular terms are mutually exclusive, reference to "or" indicates either or both possibilities. Occasionally phrases such as "and/or" are used to highlight either or both possibilities.

Reference to open-ended terms such as "comprises" allows for additional elements or steps. Occasionally phrases such as "one or more" are used with or without "comprises" to highlight the possibility of additional elements or steps.

Unless explicitly stated reference to terms such as "a" or "an" is not limited to one. For example, "a cell" does not exclude "cells". Occasionally phrases such as one or more are used to highlight the possible presence of a plurality.

Other features and advantages of the present invention are apparent from the additional descriptions provided herein including the different examples. The provided examples illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology useful for practicing the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1E provide the amino acid sequence for different HCV NS5B sequences. Figure 1A illustrates SEQ ID NO: 1, Figure 1B illustrates SEQ ID NO: 2, Figure 1C illustrates SEQ ID NO: 3, Figure 1D illustrates SEQ ID NO: 4, and Figure 1E illustrates SEQ ID NO: 5.

Figures 2A-2E provide nucleotide sequences encoding SEQ ID NO: 1-5. Figure 2A (SEQ ID NO: 6) illustrates the nucleotide sequence encoding SEQ ID NO: 1. Figure 2B (SEQ ID NO: 7) illustrates the nucleotide sequence encoding SEQ ID NO: 2. Figure 2C (SEQ ID NO: 8) illustrates the nucleotide sequence encoding SEQ ID NO: 3. Figure 2D (SEQ ID NO: 9) illustrates the nucleotide sequence encoding SEQ ID NO: 4. Figure 2E (SEQ ID NO: 10) illustrates the nucleotide sequence encoding SEQ ID NO: 5.

DETAILED DESCRIPTION OF THE INVENTION

SEQ ID NOs: 1-5 provide NS5B sequences from different HCV genotypes. SEQ ID NO: 1 is from HCV genotype 2a. SEQ ID NO: 2 is from HCV genotype 2b. SEQ ID NO: 3 is from genotype 3a. SEQ ID NO: 4 is from genotype 4a. SEQ ID NO: 5 is from genotype 6a. SEQ ID NOs: 1-5 are all modified NS5B sequences containing an amino terminus methionine and a carboxyl terminus 21 amino acid deletion.

SEQ ID NOs: 1-5 provide polypeptides having RNA-dependent RNA polymerase activity. The polypeptides have different uses, such as providing RNA-dependent RNA polymerase activity based on different sequences and being used to evaluate the ability of a compound to inhibit HCV RNA-dependent RNA polymerase activity.

The polypeptides can be used individually, or as part of a panel of RNA-dependent RNA polymerases, to evaluate the effectiveness of a compound to inhibit HCV RNA-dependent RNA polymerase activity. Compounds affecting HCV NS5B activity have research and therapeutic applications. Research applications include using the compounds as a tool to study RNA-dependent RNA polymerases activity. Therapeutic applications include using those compounds having appropriate pharmacological properties such as efficacy and lack of unacceptable toxicity to treat or inhibit onset of HCV in a patient.

NS5B Sequences

NS5B sequences described herein include polypeptides containing a region structurally related to SEQ ID NOs: 1, 2, 3, 4 or 5. A polypeptide region "structurally related" to a reference polypeptide contains an amino acid identity of at least 90% to the reference polypeptide. Polypeptides containing a region structurally related to SEQ ID NOs: 1, 2, 3, 4 or 5 can also contain additional polypeptide regions that may or may not be related to NS5B.

Percent identity to a reference sequence is determined by aligning the polypeptide sequence with the reference sequence and determining the number of identical amino acids in the corresponding regions. This number is divided by the total number of amino acids in the reference sequence (*e.g.*, SEQ ID NO: 1) and then multiplied by 100 and rounded to the nearest whole number.

Using SEQ ID NOs: 1, 2, 3, 4 or 5 as a frame of reference, alterations to the sequence can be made taking into account the known properties of amino acids. Alterations include one or more amino acid additions, deletions, and/or substitutions. The overall effect of different alterations can be evaluated using techniques described herein to confirm the ability of a particular polypeptide to provide RNA-dependent RNA polymerase activity.

Generally, in substituting different amino acids to retain activity it is preferable to exchange amino acids having similar properties. Factors that can be taken into account for an amino acid substitution include amino acid size, charge, polarity, and hydrophobicity. The effect of different amino acid R-groups on amino acid properties are well known in the art. (See, for example, Ausubel, *Current Protocols in Molecular Biology,* John Wiley, 1987-2002, Appendix 1C.)

In exchanging amino acids to maintain activity, the replacement amino acid should have one or more similar properties such as approximately the same charge and/or size and/or polarity and/or hydrophobicity. For example, substituting valine for leucine, arginine for lysine, and asparagine for glutamine are good candidates for not causing a change in polypeptide functioning.

Alterations to achieve a particular purpose include those designed to facilitate production or efficacy of the polypeptide; or cloning of the encoded nucleic acid. Polypeptide production can be facilitated through the use of an initiation codon (*e.g.,* coding for methionine) suitable for recombinant expression. Cloning can be facilitated by, for example, the introduction of restriction sites which can be accompanied by amino acid additions or changes.

Additional regions can be added to, for example, facilitate polypeptide purification or identification. Examples of groups that can be used to facilitate purification or identification include polypeptides providing tags such as a six-histidine tag, trpE, glutathione and maltose-binding protein.

In different embodiments, the SEQ ID NOs: 1, 2, 3, 4 or 5 polypeptide comprises, consists essentially, or consists, of a sequence at 90%, at least 95%, or at least 99% identical to SEQ ID NOs: 1, 2, 3, 4 or 5; or differing from SEQ ID NOs: 1, 2, 3, 4 or 5 by 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19 amino acid alterations.

Polypeptide Production and Purification

Polypeptides can be produced using standard techniques including those involving chemical synthesis and those involving purification from a cell producing the polypeptide. Techniques for chemical synthesis of polypeptides are well known in the art. (See *e.g.,* Vincent, *Peptide and Protein Drug Delivery*, New York, N.Y., Decker, 1990.)

Obtaining polypeptides from a cell is facilitated using recombinant nucleic acid techniques to produce the polypeptide. Recombinant nucleic acid techniques for producing a polypeptide involve introducing, or producing, a recombinant gene encoding the polypeptide in a cell and expressing the polypeptide.

A recombinant gene contains nucleic acid encoding a polypeptide along with regulatory elements for polypeptide expression. The recombinant gene can be present in a cellular genome or can be part of an expression vector.

The regulatory elements that may be present as part of a recombinant gene include those naturally associated with the polypeptide encoding sequence and exogenous regulatory elements not naturally associated with the polypeptide encoding sequence. Exogenous regulatory elements such as an exogenous promoter can be useful for expressing a recombinant gene in a particular host or increasing the level of expression. Generally, the regulatory elements that are present in a recombinant gene include a transcriptional promoter, a ribosome binding site, a terminator, and an optionally present operator.

Expression of a recombinant gene in a cell is facilitated through the use of an expression vector. Preferably, an expression vector in addition to a recombinant gene also contains an origin of replication for autonomous replication in a host cell, a selectable marker, a limited number of useful restriction enzyme sites, and a potential for high copy number. Examples of expression vectors are cloning vectors, modified cloning vectors, specifically designed plasmids and viruses.

Due to the degeneracy of the genetic code, a large number of different encoding nucleic acid sequences can be used to code for a particular polypeptide. The degeneracy of the genetic code arises because almost all amino acids are encoded by different combinations of nucleotide triplets or "codons". Amino acids are encoded by codons as follows:

A=Ala=Alanine: codons GCA, GCC, GCG, GCU
C=Cys=Cysteine: codons UGC, UGU
D=Asp=Aspartic acid: codons GAC, GAU
E=Glu=Glutamic acid: codons GAA, GAG
F=Phe=Phenylalanine: codons UUC, UUU
G=Gly=Glycine: codons GGA, GGC, GGG, GGU
H=His=Histidine: codons CAC, CAU
I=Ile=Isoleucine: codons AUA, AUC, AUU
K=Lys=Lysine: codons AAA, AAG
L=Leu=Leucine: codons UUA, UUG, CUA, CUC, CUG, CUU
M=Met=Methionine: codon AUG
N=Asn=Asparagine: codons AAC, AAU
P=Pro=Proline: codons CCA, CCC, CCG, CCU
Q=Gln=Glutamine: codons CAA, CAG
R=Arg=Arginine: codons AGA, AGG, CGA, CGC, CGG, CGU

S=Ser=Serine: codons AGC, AGU, UCA, UCC, UCG, UCU
T=Thr=Threonine: codons ACA, ACC, ACG, ACU
V=Val=Valine: codons GUA, GUC, GUG, GUU
W=Trp=Tryptophan: codon UGG
Y=Tyr=Tyrosine: codons UAC, UAU

Techniques for recombinant gene production, introduction into a cell, and recombinant gene expression are well known in the art. Examples of such general techniques are provided in references such as Ausubel, *Current Protocols in Molecular Biology,* John Wiley, 1987-2002, and Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual,* 2nd Edition, Cold Spring Harbor Laboratory Press, 1989.

Methods applying recombinant gene production to HCV RNA-dependent RNA polymerase expression are described in the scientific literature and the Examples provided below. The purification of full-length enzyme from insect cells transfected with a baculoviral vector has been described. (Lohmann *et al, J. Virol. 71*:8416-8428, 1997; De Francesco *et al.*, *Meth. Enzymol. 275*: 58-67, 1996). The full length enzyme has also been purified from *E. coli.* (Oh *et al, J. Virol. 73*:7694-76702, 1999).

The C-terminal region of the HCV RNA polymerase contains a stretch of highly hydrophobic amino acids that decrease the solubility of the enzyme in the absence of detergent and likely serve as a membrane anchor *in vivo*. Forms of the HCV RNA polymerase with the C-terminus truncated to remove these hydrophobic amino acids have been expressed in and purified from *E. coli* using conventional column chromatography. (Yamashita *et al, J. Biol. Chem. 273*:15479-15486, 1998; Ferrari *et al., J. Virol. 73*:1649-1654, 1999; Carroll *et al., Biochemistry 39*: 8243-8249, 2000; Luo *et al., J. Virol 74*:851-63, 2000; Leveque *et al., J. Virol. 77*:9020-9028, 2003.)

NS5B Assays

Techniques for measuring HCV RNA-dependent RNA polymerase activity are well known in the art. Examples of techniques for measuring HCV RNA-dependent RNA polymerase activity are provided in the references cited in the prior section concerning HCV expression and purification.

Examples

Examples are provided below further illustrating different features of the present invention. The examples also illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

Example 1: Rescue and Characterization of NS5B

NS5B genes were rescued and characterized from the sera of chronically infected chimpanzees. Total RNA was isolated from serum samples of chimpanzees chronically infected with HCV using the QIAGEN RNeasy Mini Kit according to manufacturer's instructions (QIAGEN, Inc. Valencia, CA). Total RNA (5 to 10 microliters) was used as a template for the reverse transcriptase reaction (Superscript II RT, Invitrogen Life Technologies, Carlsbad, CA) with a 34 nucleotide dATP primer. RT reactions were heat inactivated at 65°C for 15 minutes, and then digested with 1 μL each RNAseH and RNAseT1 (Roche Applied Science, Indianapolis, IN) at 37°C for 20 minutes to remove RNA prior to PCR. Nested PCR was performed using Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN) and the following primers:

Genotype 2a

PCR1, forward 5'-CTCCGTCGTGTGCTGCGCCATGTC (SEQ ID NO: 11)
reverse 34 nucleotide dATP (SEQ ID NO: 12)

PCR2, forward 5'TCATACTCTTGGACCGGGGCTCT (SEQ ID NO: 13)
reverse 5'GTGCCGCTCTATCGAGCGGGGAGT (SEQ ID NO: 14)

Genotype 2b

PCR1, forward 5'-ATACTCCTGGACAGGGGCCCT (SEQ ID NO: 15)
reverse 34 nucleotide dATP (SEQ ID NO: 12)

PCR2, forward 5' ATACTCCTGGACAGGGGCCCT (SEQ ID NO: 16)
reverse 5'CCGCTCTACCGAGCGGGGAGT (SEQ ID NO: 17)

Genotype 3a

PCR1, forward 5'-GAGCGTGGTCTGCTGCTCTATGTC (SEQ ID NO: 18)
reverse 5'- 34 nucleotide dATP (SEQ ID NO: 12)

PCR2, forward 5'-ATAATATGATCACACCATGTAGTGCTGAGG (SEQ ID NO: 19)
reverse 5'-CCAGCTCACCGTGCTGGCAGG (SEQ ID NO: 20)

Genotype 4a

PCR1, forward 5'-GATCGGAGGACGTCGTGTGCTGTT (SEQ ID NO: 21)
reverse 5'- 34 nucleotide dATP (SEQ ID NO: 12)

PCR2, forward 5'-GTTCGATGTCATACTCGTGGACTG (SEQ ID NO: 22)
reverse 5'-AAGCTGCCTACCGAGCAGGCAGCA (SEQ ID NO: 23)

Genotype 6a
PCR1, forward 5'-CTAAGCTCAGGCTCTTGGTCCACT (SEQ ID NO: 24)
reverse 5'- 34 nucleotide dATP (SEQ ID NO: 12)
PCR2, forward 5'-GACGACGTCGTATGTTGTTCCATG (SEQ ID NO: 25)
reverse 5'-CTACCGAGCGGGGAGCAAAAAGATG (SEQ ID NO: 26)

PCR products were cloned into pGEM-T and individual clones sequenced. Genotype was confirmed based upon closest homology to prototype sequences listed in GenBank.

Example 2: Construction of NS5B Expression Clones

The BK NS5B Δ21 gene (Carroll *et al., J. Biol. Chem. 278*:11979-11984, 2003) was modified by standard molecular biology techniques to encode the sequence Leu-Glu-His-His-His-His-His-His (SEQ ID NO: 27) (CTCGAGCACCACCACCACCACCAC SEQ ID NO: 28) at the C-terminal end of the NS5B Δ21 coding sequence after codon 570, and then followed by a stop codon. The Leu-Glu pair is encoded by a unique XhoI site that is just in front of the histidine tag. The vector was further modified to encode a unique BclI sites at NS5B codon 10. This vector served as a template to subclone additional NS5B genes for protein expression as BclI-Xho fragments.

SEQ ID NOs: 1-5 all initiate with the first 10 codons of genotype 1b BK sequences. NS5B genes were cloned in frame as BclI-XhoI fragments using clone specific PCR primers. The NS5B constructs lacked the C-terminal 21 residues, which previously was demonstrated to increase solubility. All constructs were verified by DNA sequencing.

Example 3: Bacterial Expression of NS5B Δ21 Enzymes

Glycerol stocks were used as seed cultures for large-scale purification. Glycerol stocks were prepared by transforming DNA into Rosetta™ (DE3) competent cells (Novagen). A 20 mL overnight culture of Luria-Bertani (LB) broth (containing 50 μg/mL ampicillin, 34 μg/mL chloramphenicol) was inoculated from a single colony. Cells were collected by centrifugation and used to inoculate a 1 L culture of LB broth with 100 μg/mL ampicillin only, and grown to mid-log phase (A_{600} of 0.4-0.5). To generate glycerol stocks, cells were again collected by centrifugation and resuspended, per liter of culture, in 50 mL ice cold LB broth. Then 500 μl aliquots of cells were individually mixed with 500 μl of 50% glycerol, placed into storage vials, quick frozen on dry ice and kept at -70°C until use.

For large-scale growth, a glycerol stock was plated on LB plates containing 50 μg/mL ampicillin and 34 μg/mL chloramphenicol (Teknova), incubated overnight at 37°C, collected through scraping, and used as an inoculum for a 200 mL starter culture. After ~15 minutes of shaking at 225 rpm at 37°C, 20 mL of the starter culture was used to seed 980 mL of LB broth containing 100 μg/mL ampicillin. The cultures were grown to an optical density of A_{600} nm of ~0.7, and induced with 1 mM of isopropylthio-ß-galactoside (IPTG from Invitrogen Life Technologies Inc.). The temperature and shaking were then lowered to 18°C and 210 rpm for the 18 hour induction period. Cells were collected by centrifugation and stored at -70°C until use.

Example 4: Purification of NS5B Δ21

All steps in the purification were performed on ice or in a refrigerated 4°C cold room, and with pre-chilled buffers. Cell pellets were resuspended with 200 mL of lysis buffer (20 mM Tris-HCl pH 7.5, 10% glycerol, 0.5 M KCl, 5 mM $MgCl_2$, 2 mM ß-mercaptoethanol (ß-ME), 0.2% n-octylglucoside, Complete EDTA-Free Protease Inhibitors from Roche Diagnostics Corp.). To this was added 5,000U DNase I (grade I, Roche) and incubated with stirring for 10 minutes. This mixture was dounce homogenized until the lysate was homogenous, then fluidized with three passes thru the Microfluidizer (model 110Y, Microfluidics Corporation). The fluidized lysate was centrifuged at 15,000 rpm for 30 minutes in a JA-17 rotor (Beckman Coulter).

The supernatant was collected, mixed with 5 mL of packed TALON® CellThru resin (Cobalt affinity resin, Clontech), and incubated for 1 hour with gentle agitation to allow sample binding. The mixture was centrifuged at 1750 rpm in the GH-3.8 rotor (Beckman Coulter) for 5 minutes to pellet the resin. The protein-bound resin was washed with 5 column volumes of Wash-EQ buffer (20 mM Tris-HCl pH 7.5, 10% glycerol, 0.5 M KCl, 2 mM ßME, 0.2% n-octylglucoside) for 5 minutes, the resin pelleted by centrifugation at 1750 rpm in the GH-3.8 rotor for 2 minutes, and the supernatant removed. This wash procedure was repeated an additional four times. The resin was then washed a final time with 5 column volumes of Wash buffer (20 mM Tris-HCl pH 7.5, 10% glycerol, 0.5 M KCl, 2 mM ßME, 0.2% n-octylglucoside, 10 mM Imidazole).

To elute protein, the resin was resuspended with 1 column volume of elution buffer (20 mM Tris-HCl pH 7.5, 10% glycerol, 0.5 M KCl, 2 mM ß-ME, 0.2% n-octylglucoside, 200 mM Imidazole) and incubated with gentle agitation for 10 minutes. The resin was pelleted by centrifugation at 1750 rpm in the GH-3.8 rotor for 2 minutes, the eluate collected, and EDTA added to a final concentration of 1 mM. The elution procedure was repeated twice more, but the

eluates were kept separate. The eluates were then dialyzed in dialysis buffer (20 mM Tris-HCl pH 7.5, 10% glycerol, 0.5 M KCl, 3 mM dithiothreitol (DTT), 0.2% n-octylglucoside) with a change of buffer. Concentrated eluate fractions (> 50 % of the most concentrated fraction) were combined, aliquoted, quick frozen on dry ice, and stored enzyme at -70°C until use.

Protein quantitation was performed using Pierce's Coomassie Plus Protein reagent and Molecular Devices Spectra Max 250 with the SOFTmaxPRO v3.1.1 software. Protein visualization was performed using 4-15% gradient Tris-HCl SDS PAGE gels (Bio-Rad) and Bio-Safe Coomassie (Bio-rad). Protein purity was determined by quantitation using the Storm860 and ImageQuant software (Molecular Dynamics).

Example 5: Polymerase Assay

The genotype 2a (SEQ ID NO: 1), 2b (SEQ ID NO: 2), and 3a (SEQ ID NO: 3) polymerases were titrated in activity-linearity assays in a final concentration range between 62.5 nM to 1500 nM (1250 nM for the SEQ ID NO: 3 enzyme). Polymerase was pre-incubated for 1 hour at room temperature with 0.75 μg per reaction of t500 RNA template (IBA GMBH) in a volume of 45 μl. t500 RNA template is comprised of bases 3504-4004 of the HCV BK genome and corresponds to the NS2/3 region as previously described (Carroll *et al., Biochemistry 39*:8243-8249, 2000). The following final buffer conditions were: 20 mM Tris-HCl pH 7.5; 50 μM EDTA; 5 mM DTT; 2 mM $MgCl_2$; 80 mM KCl; 0.4 U/μL rRNAsin (Promega).

The reaction was initiated by the addition of 5 μl of a nucleotide triphosphate cocktail which consisted of 10 μM each ATP, CTP, UTP, and GTP (Ultrapure NTP set from Amersham Biosciences) which had been spiked with 0.2 μl of α^{33}P GTP (10 mCi/ml, Perkin Elmer Life Sciences). Assay conditions for genotype 4a (SEQ ID NO: 4) and 6a (SEQ ID NO: 5) enzymes were identical to that described for SEQ ID NOs: 1-3 except that the nucleotide concentrations were 100 μM each. The final enzyme reaction volume was 50 μl. To quench the reaction, 20 μL of 0.5 M EDTA was added. For quantitation, 50 μL of the quenched reaction was blotted onto DE81 Whatman filter disks, dried, washed ten times with 200 mL of 0.3 M ammonium formate pH 8.0, ethanol rinsed, dried, imaged with Storm860/ImageQuant, and quantitated by liquid scintillation counting. The results are shown in Tables 1 and 2. By way of comparison, a Δ21 histidine tagged HCV BK NS5B purified and assayed under similar conditions had a specific activity of 74 nmol/ hr*mg.

Table 1

SEQ ID NO:	Specific Activity [nmol/(hr*mg)]
1	2
2	15
3	147

Table 2

SEQ ID NO:	Specific Activity [nmol/(hr*mg)]
4	2
5	2

Other embodiments are within the following claims. While several embodiments have been shown and described, various modifications may be made without departing from the spirit and scope of the present invention.